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Potential sources of high value chemicals from leaves, stems and flowers of *Miscanthus sinensis* 'Goliath' and *Miscanthus sacchariflorus*

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ABSTRACT

Society demands chemicals from sustainable sources. Identification of commercially important chemicals in crops increases value in biorefineries and reduces reliance on petrochemicals. *Miscanthus sinensis* and *Miscanthus sacchariflorus* are high-yielding distinct plants, which are sources of high-value chemicals and bioethanol through fermentation. Cinnamates in leaves, stems and flowers were analysed by LC-ESI-MS^n. Free phenols were extracted and separated chromatographically. More than twenty hydroxycinnamates were identified by UV and LC-ESI-MS^n. Several cinnamate hexosides were detected in the *M. sinensis* flower and in *M. sacchariflorus* (leaf and stem). Hydroxybenzoic acids and their hexosides were observed in leaf and stem of *M. sacchariflorus*. Higher concentrations of 3-feruloylquinic acid were observed in *M. sacchariflorus* stem, suggesting a role in cell-wall biosynthesis. This technique can be used to screen plants in a mapping family to identify genotypes/species with high concentrations of phenols. Plants with low concentrations of antimicrobial phenols may be good feedstocks for fermentation.

1. Introduction

Concerns about global warming and depletion of fossil fuels have stimulated interest in development of cleaner technologies that use sustainable and carbon-neutral feedstocks (Alonso et al., 2010; Sims et al., 2006). Sustainable feedstocks will be provided, at least in part, by the cultivation of non-food energy crops (Lewandowski et al., 2000; Sims et al., 2006). *Miscanthus* is a genus consisting of ca. fifteen species of perennial grasses that are native to tropical and subtropical regions of Africa, South Asia and temperate zones of Asia (Villaverde et al., 2010a,b). *Miscanthus* × *giganteus* (*M.* × *giganteus*), a perennial rhizomatous grass with C_4-photosynthesis, can grow to heights of more than 3.5 m in a single growth season (Villaverde et al., 2010a,b). This hybrid is a sterile triploid formed by a cross between *Miscanthus sacchariflorus* (*M. sacchariflorus*) and *Miscanthus sinensis* (*M. sinensis*). In this paper, we evaluate and compare the phenolic composition of these two claimed parent species of this hybrid.

There is much interest in using *Miscanthus* as an alternative to food crops and petroleum-based feedstocks to make a variety of bulk, intermediate and speciality chemicals, in addition to providing fuel. *Miscanthus* plants can grow under a range of climatic and environmental conditions (Lewandowski et al., 2000). After senescence, the plant can be burned for heat and electricity or fermented to produce biofuel. The chemical composition can affect the efficiency of conversion of biomass to energy and to chemical products (Klinke et al., 2004). Soluble phenols inhibit the bioconversion of sugars to ethanol, as many are toxic to the fermenting microorganisms (Klinke et al., 2004); thus it is important to profile the content of these unwanted components.

Abbreviations: ADME, absorption, distribution, metabolism, excretion; CADPE, 2-(3,4-dihydroxyphenyl)ethyl caffeate; CAPE, 2-phenylethyl caffeate; 2-CaffHydCitA, 2-caffeoylhydroxycitric acid; 3-*p*-CoQA, 3-*para*-coumaroylquinic acid; 4-*p*-CoQA, 4-*para*-coumaroylquinic acid; 5-*p*-CoQA, 5-*para*-coumaroylquinic acid; CaffQA, caffeoylquinic acid; 1-CaffQA, 1-caffeoylquinic acid; 3-CaffQA, 3-caffeoylquinic acid; 4-CaffQA, 4-caffeoylquinic acid; 5-CaffQA, 5-caffeoylquinic acid; CaffSA, caffeoylshikimic acid; 3-CaffSA, 3-caffeoylshikimic acid; 4-CaffSA, 4-caffeoylshikimic acid; 5-CaffSA, 5-caffeoylshikimic acid; 2-CaffTA, 2-caffeoylthreonic acid; 2,3-diHydBA, 2,3-dihydroxybenzoic acid; 2,5-diHydBA, 2,5-dihydroxybenzoic acid; 3,4-diHydBA, 3,4-dihydroxybenzoic acid; 2,3-diHydBAHex, 2,3-dihydroxybenzoic acid hexoside; 3,4-diHydBAHex, 3,4-dihydroxybenzoic acid hexoside; 1,4-diCaffQA, 1,4-dicaffeoylquinic acid; 1,5-diCaffQA, 1,5-dicaffeoylquinic acid; 3,4-diCaffQA, 3,4-dicaffeoylquinic acid; 3,5-diCaffQA, 3,5-dicaffeoylquinic acid; FA, ferulic acid; 2-FHydCitA, 2-feruloyl hydroxycitric acid; 3-FQA, 3-feruloylquinic acid; 4-FQA, 4-feruloylquinic acid; 5-FQA, 5-feruloylquinic acid; 2-FTA, 2-O-feruloylthreonic acid; FW, fresh weight; 2-HydBA, 2-hydroxybenzoic acid; 3-HydBA, 3-hydroxybenzoic acid; 4-HydBA, 4-hydroxybenzoic acid; HydBAHex, hydroxybenzoic acid hexoside; HIV-1, human immunodeficiency virus-1; HPLC, high-performance liquid chromatography; LC-ESI-MS, liquid chromatography-electrospray ionisation mass spectrometry; *p*-CoA, *para*-coumaric acid; PDA, photodiode array; SA, syringic acid; UV, ultra-violet.

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Plants produce a wide range of monophenolic and polyphenolic compounds with roles in strengthening cell walls, protection against UV radiation, tolerance to stress and resistance to pathogens. Hydroxycinnamic acids are the most widely distributed group of secondary compounds and are present as free, conjugated-soluble and bound-insoluble forms. The acids are often found as glycosides, sugar esters and amides. Caffeate esters predominate in grass, the most abundant being 5-*O*-caffeoylquinic acid. Hydroxycinnamic acids (specifically ferulic and *p*-coumaric acids) are covalently linked into the cell wall of grass species, where they cross link hemicellulose and lignin.

The potential benefits of these compounds are now appreciated by food and cosmetic industries (Dimitrios, 2006; Naczk and Shahidi, 2004, 2006; Ou and Kwok, 2004; Padilla et al., 2005). Pharmaceutical companies continue to use natural products as sources of leads for the development of drugs (Harvey, 2008; Ou and Kwok, 2004) and hydroxycinnamic acid conjugates are considered important drug leads (Touaibia et al., 2011). Many are reducing agents, hydrogen-donating antioxidants and quenchers of singlet oxygen (Parveen et al., 2011); thus diverse phenolic derivatives have interesting biological activities (Touaibia et al., 2011).

We recently reported the use of LC-ESI-MS^n to profile rapidly more than twenty phenols in the leaf and stem tissues of the hybrid *M.* × *giganteus,* including several novel mandelonitriles and mandelamides (Parveen et al., 2011). Total phenol concentration (hydroxycinnamates) in the leaf tissue was 0.04% FW and in the stem tissue 0.02% FW. The analysis was carried out on green material when the plant was at its highest biomass. Here we use LC-ESI-MS^n for qualitative and quantitative profiling of hydroxycinnamates in leaf, stem and flower extracts of the parental lines *M. sinensis* and *M. sacchariflorus* at the same high biomass stage of growth as was *M.* × *giganteus*, to draw comparisons and conclusions about the relative utilities of these plants. Knowledge of the content and composition of the soluble phenol chemistry of these plants would help to determine the potential for exploiting this plant.

2. Results and discussion

2.1. Extraction of phenols

Soluble phenols were obtained from the methanol extract of fresh leaves, stems and flowers of *M. sinensis* and leaves and stems of *M. sacchariflorus*. More than twenty phenols (including hydroxycinnamic acid conjugates) were identified with UV absorptions typically in the region (240–340 nm) (Tables 2 and 3 (Supplementary Information) and Figs. 1 and 2). The UV absorbance maxima could not be recorded for some cinnamic acid conjugates, either owing to low abundance or because they were masked by co-eluting peaks. The identification method used UV absorption coupled with the fragmentation pattern observed in tandem mass spectra using LC-ESI-MS^n. Where possible, the mass spectra and retention times of the phenols were compared with those of standards, including caffeic acid, *o*-coumaric acid, *p*-coumaric acid, cinnamic acid, ferulic acid, syringic acid, 5-caffeoylquinic acid (5-CaffQA, also known as chlorogenic acid), sinapic acid, 2-, 3-, and 4-hydroxybenzoic acid (2-HydBA, 3-HydBA and 4-HydBA), 2,3, 2,5- and 3,4-dihydroxybenzoic acid (2,3-diHydBA, 2,5-diHydBA and 3,4-diHydBA), 2-hydroxy-3-methoxybenzoic acid, 2-hydroxy-4-methoxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid (vanillic acid). In the absence of commercial standards, caffeoyl-, *p*-coumaroyl- and feruloyl-conjugates were assigned primarily by their parent ion; their UV spectrum and elution order and assignments were supported by comparison of their mass spectrometric fragmentation data (MS^2 and MS^3) to those previously reported (Clifford et al., 2005; Jaiswal et al., 2010; Parveen et al., 2008). In the absence of standards, all other identifications are considered provisional.

2.2. Characterisation of caffeoyl derivatives

This is the first study on the HPLC profiles of phenols in *M. sinensis* and *M. sacchariflorus* tissues. In all tissues, the most abundant compound was 5-*O*-caffeoylquinic acid (5-CaffQA). Particularly high concentrations of 3-*O*-caffeoylquinic acid (3-CaffQA) were detected in *M. sacchariflorus* tissues (Tables 1–3 (Supplementary Information)). The pattern of fragmentation and retention time for 5-CaffQA were consistent with those of a commercial standard. Diagnostic fragmentation ions of caffeoylquinic acids in negative-ion mode ESI-MS^n involved one of two pathways (i) loss of the acyl group with cleavage of the carbonyl-oxygen bond, (ii) β-elimination of a carboxylic acid (Parveen et al., 2011). Geometrical isomers were evident; for example, *cis*/*trans* isomers of caffeic acid were found in all extracts, based on photoirradiation experiments resulting in photo-isomerisation (Parveen et al., 2011). As observed previously in the stem extract of *M. sinensis*, a small but distinct peak showed properties very similar to that of 5-CaffQA in MS/MS negative-ion mode spectra. This was evidently a CaffQA-stereoisomer but 1-CaffQA was discounted, as this peak did not co-elute with an authentic sample of 1-CaffQA from acid-catalysed hydrolysis of cynarin (1,3-dicaffeoylquinic acid) (Parveen et al., 2011).

In *M. sinensis* leaf and *M. sacchariflorus* leaf and stem tissues, minor compounds giving peaks at *m*/*z* 335 [M−H]$^-$ and ions at *m*/*z* 317, 291, 179, 161, 135 were consistent with *O*-caffeoylshikimic acids (CaffSA) (Jaiswal et al., 2010). As for quinic acid, shikimic acid can form esters with cinnamic acids. Jaiswal et al. (2010) reported the synthesis and fragmentation pathways of 3-, 4-, and 5-CaffSA in negative ion mode. CaffSAs have been widely reported in plants (Fang et al., 2002; Jaiswal et al., 2010); however, they were minor components in the *Miscanthus* tissue extracts. Three minor components with *pseudo*molecular ion 515 (M−H)$^-$ were detected in the leaf of *M. sacchariflorus* and were identified by their fragmentations patterns as 1,4-diCaffQA, 3,4-diCaffQA and 3,5-diCaffQA (Clifford et al., 2007). Dicaffeoylquinic acids have previously been reported in *M.* × *giganteus* and in herbal chrysanthemum (Clifford et al., 2007; Parveen et al., 2011). Three further compounds in *M. sinensis* flowers and leaf and stem of *M. sacchariflorus* with *pseudo*molecular ion 515 [M−H]$^-$ were clearly hydroxycinnamates but not dicaffeoylquinic acids. The MS^2 experiment yielded ions *m*/*z* 353, 341, 323, 191 and 179. These compounds were tentatively identified as caffeoylquinic acid hexosides where the hexose is linked to a hydroxy on a caffeoyl moiety as a glycoside; the linkage hexose-caffeoyl-quinic acid is demonstrated by the observation of fragment ions corresponding to CaffQAs. These compounds were previously reported in the hybrid *M.* × *giganteus* (Parveen et al., 2011). In *M. sacchariflorus,* compounds showing *pseudo*molecular ions and fragmentation patterns in negative-ion mode *m*/*z* 297 [M−H]$^-$ and 369 [M−H]$^-$ corresponded to 2-*O*-caffeoylthreonic acid (2-CaffTA) and 2-*O*- acid caffeoyloxycitric (2-CaffHydCitA), respectively. These compounds have previously been reported in *Dactylis glomerata* and in *Cornus controversa* (Lee et al., 2000; Parveen et al., 2011). Other caffeoyl-derivatives were observed in *M. sacchariflorus* but these could not be identified. Examples of structures are shown in Fig. 3.

2.3. Characterisation of p-coumaroyl derivatives

The patterns of fragmentation observed at MS^2 and MS^3 enabled the identification of three *trans*- isomers of *O*-*p*-coumaroylquinic acid (3-*p*-CoQA, 4-*p*-CoQA and 5-*p*-CoQA) in *M. sinensis* and *M. sacchariflorus* tissue extracts. The 5-acylated isomer was characterised by a MS^2 base ion *m*/*z* 191; 3-*p*-CoQA yielded MS^2 base peak *m*/*z* 163 [coumarate]$^-$ and 4-*p*-CoQA was distinguished by β-elimination of coumaric acid to give MS^2 base ion *m*/*z* 173. Several isomers with *pseudo*molecular ions 325 [M−H]$^-$, MS^2 base ion